

Characterization of a hormonogenic domain from human thyroglobulin

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A polypeptide domain of molecular mass near 22 kDa was purified from CNBr-digest of iodine poor human thyroglobulin (hTgb). This fragment represents the N-terminal part of the hTgb molecule and consequently contains the preferential hormonogenic tyrosine 'acceptor' of the protein. This fragment could correspond to the non-iodinated and unreduced form of the thyroxinyl-containing 26 kDa peptide previously purified from reduced and iodinated hTgb. This 22 kDa fragment is capable by itself, i.e. independently of the remaining hTgb molecule, of synthesizing thyroxine with a high efficiency after in vitro iodination. Its study should constitute a valuable way to identify at least one of the hormonogenic tyrosine 'donor' residues of hTgb.

(Human) Thyroglobulin Hormonogenic domain Iodination

1. INTRODUCTION

Thyroglobulin (Tgb), the dimeric glycoprotein (2×330 kDa) of the thyroid gland, is the support of thyroid hormone synthesis, T_4 and T_3 . In the course of hormone formation, the coupling of two iodotyrosine residues results in the fission of one of them next to its aromatic ring and the iodophenolic moiety of the iodotyrosine residue which undergoes fission (donor) is transferred to the other iodotyrosine residue (acceptor). Recent knowledge of the primary structure of Tgb [1–3] and of the amino acids flanking the thyroid hormone residues in the mature protein made possible the precise enumeration and localization of the hormone forming (acceptor) sites of Tgb [4–7]. In contrast the position of the iodotyrosine donor

residues remains elusive. It is evident that the spatial alignment, the distance and the orientation of the interacting tyrosine residues (acceptor and donor) are the most important factors.

Recently the hypothesis that the Tgb molecule is arranged in a domain-like structure and that both hormonogenic tyrosine residues (acceptor + donor) are located in a self-contained domain which may be functionally independent of the remaining sequence has been suggested [8].

In this report we describe the isolation of a hormonogenic domain from human Tgb (hTgb); this fragment corresponding to the N-terminal part of the molecule contains the preferential hormonogenic tyrosine residue acceptor of hTgb and is capable by itself of synthesizing T_4 with high efficiency after in vitro iodination.

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Abbreviations: hTgb, human thyroglobulin; MIT, 3-iodotyrosine; DIT, 3,5-diiodotyrosine; T_4 , thyroxine; T_3 , 3,5,3'-triiodothyronine

2. MATERIALS AND METHODS

2.1. Thyroglobulin preparation

Poorly iodinated hTgbs were purified from colloid goitres as in [9]. Very iodine-rich hTgb was

prepared from a poorly iodinated hTgb after *in vitro* iodination as in [7] using the amount of KI necessary to incorporate about 70 I atoms/molecule. The iodoamino acid composition of an iodine poor and an iodine rich hTgb is given in table 3.

2.2. Cyanogen bromide (CNBr) cleavage

For each preparation 300 mg hTgb were treated by CNBr as in [10]; the resulting CNBr-peptides were filtered onto a Sephadex G-200 column in 1 M propionic acid. Each fraction was dialyzed against redistilled water and freeze-dried.

2.3. *In vitro* iodination of 22 kDa species

In a final volume of 500 μ l of 0.05 M Tris-HCl, pH 7.2, at 37°C were added 10 nmol 22 kDa species, 1 mg glucose, from 40 to 180 nmol KI and 5 μ g lactoperoxidase (Boehringer Mannheim, FRG). The reaction was initiated by 2.5 μ g glucose oxidase (Boehringer) and stopped 30 min later by addition of NaHSO₃. Excess iodide was eliminated by dialysis against redistilled water. Iodine incorporated was estimated by iodoamino acid analysis after total enzymic hydrolysis as in [11]. A maximal hormonesynthesis efficiency was obtained using 14 nmol KI/nmol peptide.

2.4. Disulfide bond estimation

The number of disulfide bonds was calculated from the difference between total half-cystine residues estimated as carboxymethylcysteine residues after reduction and S-carboxymethylation and free cysteine residues measured after S-carboxymethylation without previous reduction.

2.5. Other techniques

Reduction and S-alkylation of 22 kDa species were performed as in [10] except that the reagents were eliminated by Biogel P-2 filtration in 0.1 M ammonium bicarbonate. SDS-PAGE, amino acid and iodoamino acid analyses and N-terminal sequence determination of peptides were carried out as described [4,12].

3. RESULTS AND DISCUSSION

3.1. Purification of 22 kDa species

The elution profile of Sephadex G-200 gel filtration (fig.1) of CNBr-cleaved iodine poor (A) and

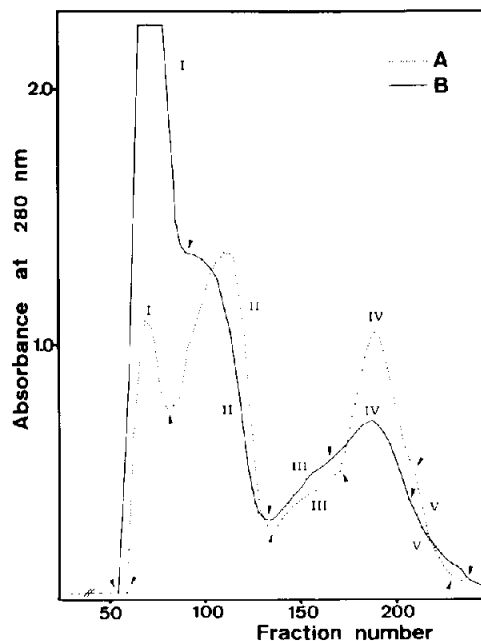


Fig.1. Sephadex G-200 gel filtration of CNBr-treated iodine poor (A) and iodine rich (B) hTgb. 300 mg protein were layered onto a 2.6 \times 90 cm column equilibrated and eluted with 1 M propionic acid. Flow rate, 6 ml/h; volume of fractions, 2 ml. Fractions were pooled according to arrowheads, to give fractions I–V.

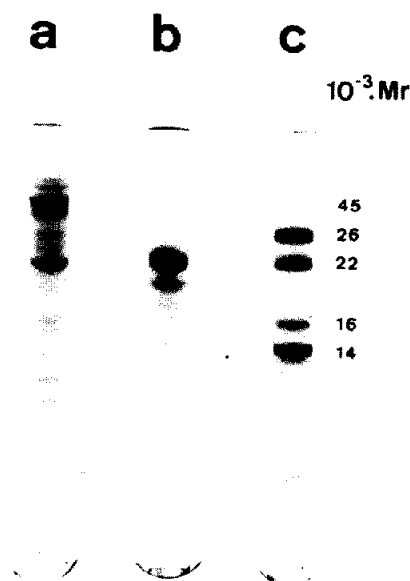


Fig.2. SDS-PAGE of (lane a) fraction IV (see fig.1A) and (lane b) purified 22 kDa species before and (lane c) after reduction by 1% dithiothreitol for 3 min at 100°C.

Proteins were analyzed in 15% gels.

very iodine-rich (B) hTgb was different: indeed, in A fraction IV was higher and, inversely, fraction I (containing heavier material) lower than in B. These results suggest that a part of protein material from fraction IV shifted towards fraction I after iodination and hormonesynthesis. Qualitatively the same species were found in fraction IV whatever the origin of hTgb (poorly iodinated or very iodine-rich hTgb); SDS-PAGE revealed essentially a group of bands with molecular masses near 45 kDa and a 22 kDa species (fig.2). In an attempt to purify further the 22 kDa component, material in fraction IV was applied on a Biogel A-0.5m column and eluted with 0.1 M ammonium bicarbonate. The 22 kDa component was obtained with small amounts of a lighter species; in spite of a new filtration on Biogel P-30 this contaminant could not be quite eliminated.

3.2. Characterization of 22 kDa species

After reduction of the 22 kDa component, SDS-PAGE showed (fig.2) 4 new bands of molecular masses near 26, 22, 16 and 14 kDa. So, a 22 kDa species purified from CNBr-treated iodine poor hTgb was submitted to N-terminal sequence analysis: two residues with small amounts of a third one were unambiguously identified at almost each step (table 1, row I). From these results, the three deduced amino acid sequences perfectly agreed with: (i) the N-terminal sequence of hTgb; (ii) the amino acid sequence located after the methionine residue 127 and (iii) the sequence after the methionine 184 (fig.3).

In table 2 the amino acid composition of the 22 kDa species is compared with that of the 26 kDa hormonepeptide previously purified from iodinated, fully reduced and S-alkylated hTgb

Table 1
Micro-sequencing of 22 kDa species

Degradation steps		No.: 1	2	3	4	5	6
I	22 kDa species from iodine poor hTgb	Asn Glu Ile (tr)	Ile Val Phe (tr)	Phe Tyr Asp	Glu Gly Leu (tr)	Tyr Thr	Gln Arg
II	22 kDa species from iodine rich hTgb	Asn/Asp Glu Ile (tr)	Ile Val Phe (tr)	Phe Tyr (tr) Asp (tr)	Glu Gly	MIT/DIT/T ₄ Thr	
III	22 kDa species from iodine poor hTgb and after in vitro iodination	Asn/Asp Glu Ile (tr)	Ile Val Phe (tr)	Phe Tyr (tr) Asp (tr)	Glu Gly	DIT/T ₄	

Micro-sequencing by technique of Chang et al. [12]; tr, traces

1 Asn-Ile-Phe-Glu-Tyr-Gln-Val-Asp-Ala-Gln-Pro-Leu-Arg-Pro-Cys-Glu-Leu-Gln-Arg-Glu-Thr-Ala-Phe-Leu-Lys-Gln-Ala-Asp-Tyr-Val-
 31 Pro-Gln-Cys-Ala-Glu-Asp-Gly-Ser-Phe-Gln-Thr-Val-Gln-Cys-Gln-Asn-Asp-Gly-Arg-Ser-Cys-Trp-Cys-Val-Gly-Ala-Asn-Gly-Ser-Glu-
 61 Val-Leu-Gly-Ser-Arg-Gln-Pro-Gly-Arg-Pro-Val-Ala-Cys-Leu-Ser-Phe-Cys-Gln-Leu-Gln-Lys-Gln-Gln-Ile-Leu-Leu-Ser-Gly-Tyr-Ile-
 91 Asn-Ser-Thr-Asp-Thr-Ser-Tyr-Leu-Pro-Gln-Cys-Gln-Asp-Ser-Gly-Asp-Tyr-Ala-Pro-Val-Gln-Cys-Asp-Val-Gln-His-Val-Gln-Cys-Trp-
 121 Cys-Val-Asp-Ala-Glu-Gly-Met-Glu-Val-Tyr-Gly-Thr-Arg-Gln-Leu-Gly-Arg-Pro-Lys-Arg-Cys-Pro-Arg-Ser-Cys-Glu-Ile-Arg-Asn-Arg-
 151 Arg-Leu-Leu-His-Gly-Val-Gly-Asp-Lys-Ser-Pro-Pro-Gln-Cys-Ser-Ala-Glu-Gly-Glu-Phe-Met-Pro-Val-Gln-Cys-Lys-Phe-Val-Asn-Thr-
 181 Thr-Asp-Met-Met-Ile-Phe-Asp-Leu-Val-His-Ser-Tyr-Asn-Arg-Phe-Pro-Asp-Ala-Phe-Val-Thr-Phe-Ser-Ser-Phe-Gln-Arg-Arg-Phe-Pro-
 211 Glu-Val-Ser-Gly-Tyr-Cys-His-Cys-Ala-Asp-Ser-Gln-Gly-Arg-Glu-Leu-Ala-Glu-Thr-Gly-Leu-Glu-Leu-Leu-Leu-Asp-Glu-Ile-Tyr-Asp-
 241 Thr-Ile-Phe-Ala-Gly-Leu-Asp-Leu-Pro-Ser-Thr-Phe-Thr-Glu-Thr-Thr-Leu-Tyr-Arg-Ile-

Fig.3. Sequence of the first 260 amino acids from the N-terminal end of mature hTgb as deduced from the nucleotide sequence of its mRNA according to Malthiery and Lissitzky [2]. The tyrosine residue in position 5 (•) is the hormonogenic residue (acceptor) described [7]. Methionine residues are boxed.

Table 2

Comparison of the 22 kDa species amino acid composition with that of the 26 kDa hormonepeptide

	22 kDa species ^a	26 kDa hormonepeptide according to	
		Lejeune et al. [7]	Dunn et al. [13]
Carboxymethylcysteine ^b	14.2	12.6	11.0
Aspartic acid	21.3	18.0	23.3
Threonine	9.1	7.1	10.7
Serine	18.7	15.3	15.9
Glutamic acid	40.7	37.8	39.6
Proline	12.3	12.1	12.9
Glycine	21.0	16.5	18.3
Alanine	14.6	14.0	19.8
Cysteine ^c	0.6		
Valine	16.5	16.2	18.8
Methionine	1.2	2.2	1.7
Isoleucine	6.0	6.1	6.7
Leucine	18.8	16.3	17.6
Tyrosine	5.9	6.4	3.8
Phenylalanine	10.2	8.2	10.2
Histidine	2.6	1.9	5.0
Lysine	7.3	5.1	11.4
Arginine	11.8	8.8	11.4
Tryptophan	nd	nd	nd

^a Average of analyses from 6 separate preparations of 22 kDa species

^b Determined after reduction and S-carboxymethylation

^c Determined as carboxymethylcysteine after S-carboxymethylation without previous reduction (see section 2.4)

Results expressed as residue/mol peptide, all amino acid compositions were calculated assuming a molecular mass of 26 kDa; nd, not determined

[7,13]. It has been shown that this hormonepeptide represents the N-terminal part of the hTgb molecule and contains at the fifth position from the N-terminal asparagine residue (fig.3) the preferential hormonogenic tyrosine acceptor of hTgb [7]. When the amino acid composition of 22 kDa species was calculated on the basis of a molecular mass of 26 kDa, both compositions agreed reasonably well.

Taking into account these results, it is likely that the 22 kDa represents the N-terminal part of hTgb; moreover it could correspond to the non-iodinated

and unreduced form of 26 kDa hormonepeptide. However it is difficult to affirm this latter hypothesis since the C-terminal sequence has never been clearly identified either in the 26 kDa [14] or 22 kDa (not shown).

The N-terminal amino acid sequence of hTgb shown in fig.3 contains several methionyl bonds. In 22 kDa species some of them are partially (or totally) cleaved by CNBr and the derived CNBr-peptides are disulfide bonded. Indeed nearly all of the half-cystine residues contained in 22 kDa (table 2) are involved in disulfide bonds. So, after reduction 22 kDa gives rise to several peptides (fig.2, lane c), among which 26 kDa could represent the reduced and consequently unfolded form of the uncleaved 22 kDa fragment; indeed as previously described for several proteins rich in disulfide bonds [15,16] the shift in molecular mass after reduction results from the unfolding of disulfide bonded internal domains leading to an apparent increase of chain length.

3.3. Hormonosynthesis in 22 kDa species

When 22 kDa was purified from very iodine-rich hTgb, it contained 0.51 mol T₄/mol peptide (table 3) and at the fifth step of recurrent degradation MIT, DIT and T₄ were identified (table 1, row II). In contrast, when purified from iodine poor hTgb, 22 kDa contained small amounts of MIT, traces of DIT and no T₄; but after in vitro iodination under optimal conditions (see section 2.3) it is capable of synthesizing T₄ with efficiency (table 3). In addi-

Table 3

Iodoamino acid composition of iodine poor (hTgb A), iodine rich (hTgb B) human thyroglobulin and their derived 22 kDa species

	MIT	DIT	T ₃	T ₄
hTgb A	2.82	0.28	0	tr
22 kDa from hTgb A ^a	0.40	tr	0	0
22 kDa from hTgb A ^a after in vitro iodination	0.70	1.10	0	0.23
hTgb B	27.5	17.8	0	2.0
22 kDa from hTgb B	0.60	1.10	0	0.51

^a Average of 6 separate experiments

Results expressed as residue/mol, determined assuming a molecular mass of 660 kDa for hTgb and 26 kDa for 22 kDa species; tr, traces

tion after micro-sequencing of the in vitro iodinated 22 kDa, DIT and T₄ were identified at the fifth step (table 1, row III). It is obvious that in the course of the coupling reaction which occurs either in 22 kDa peptide or in the whole hTgb molecule the same tyrosine residue acceptor is involved in T₄ synthesis. In spite of the partial cleavage of some (methionyl or others) bonds in the 22 kDa, it is possible that this fragment owing to its high degree of cross-linkage (about 7 disulfide bonds; table 2) has retained an appreciable part of the three-dimensional native conformation allowing this hormonogenic domain to synthesize T₄ in vitro with efficiency.

The localization of the tyrosine residue donor involved in T₄ formation in this domain is the aim of our present investigations.

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